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Efficacy of Commercial Vaccines in Protecting Chickens and Ducks Against H5N1 Highly Pathogenic Avian Influenza Viruses from Vietnam

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SUMMARY. Highly pathogenic (HP) H5N1 avian influenza (AI) viruses continue to circulate in Asia and have spread to other regions of the world. Though attempts at eradication of the viruses during various outbreaks have been successful for short periods of time, new strains of H5N1 viruses continue to emerge and have become endemic in parts of Asia and Africa. Vaccination has been employed in Vietnam as part of AI control programs. Domestic ducks, which make up a large part of poultry in Vietnam, have been recognized as one of the primary factors in the spread of AI in this country. As a result, ducks have been included in the vaccination programs. Despite the effort to control AI in Vietnam, eradication of the disease has not been possible, due in part to the emergence and spread of new viruses. Here, we tested the abilities of avian influenza oil emulsion vaccines of different genetic origins to protect against disease and viral shedding in both 2-wk-old white leghorn chickens and 1-wk-old Pekin ducks. Seventy-five to 100% of vaccinated chickens were protected from mortality, but viral shedding occurred for at least 4 days post challenge. All but one vaccinated duck were protected from mortality; however, all groups shed virus up through at least 5 days postchallenge, depending on the vaccine and challenge virus used. Differences in levels of hemagglutination inhibition (HI) antibody titers induced by the vaccines were observed in both chickens and ducks. Although the vaccines tested were effective in protecting against disease and mortality, updated and more efficacious vaccines are likely needed to maintain optimal protection.

RESUMEN. Eficacia de vacunas comerciales para proteger a pollos y patos contra virus de la influenza aviar de alta patogenicidad H5N1 originarios de Vietnam.

Los virus de la influenza aviar H5N1 de alta patogenicidad continúan circulando en Asia y se han diseminado a otras regiones del mundo. Aunque los intentos para erradicar estos virus durante varios brotes han sido exitosos por periodos de tiempo cortos, nuevas cepas de los virus H5N1 continúan emergiendo y se han convertido en virus endémicos en partes de Asia y África. La vacunación ha sido utilizada en Vietnam como parte de programas de control. Los patos domésticos, que constituyen una gran parte de la avicultura en Vietnam, se han identificado como uno de los factores primarios en la diseminación de la influenza aviar en este país. Como resultado, los patos han sido incluidos en los programas de vacunación. A pesar de los esfuerzos para el control de influenza aviar en Vietnam, la erradicación de la enfermedad no ha sido posible, debido en parte a la aparición y diseminación de virus nuevos. En este trabajo, se estudió la capacidad de las vacunas contra la influenza aviar emulsionadas en aceite con diferentes orígenes genéticos para proteger contra la enfermedad y contra la eliminación del virus en aves Leghorn blancas de dos semanas de edad y en patos Pekín de una semana de edad. Del 75% al 100% de los pollos vacunados estuvieron protegidos contra la mortalidad, pero la eliminación viral se presentó por lo menos durante cuatro días después del desafío. Con excepción de un pato, casi todos los patos vacunados estuvieron protegidos contra la mortalidad, sin embargo, todos los grupos eliminaron al virus por lo menos durante cinco días después del desafío dependiendo de la vacuna y del virus de desafío aplicados. Se observaron diferencias en los niveles de anticuerpos inhibidores de la hemoaglutinación inducidos por las vacunas en los pollos y en los patos. Aunque las vacunas estudiadas fueron efectivas para proteger contra la enfermedad y mortalidad, probablemente se requieren vacunas más eficaces y actualizadas para mantener una protección óptima.

Key words: avian influenza, vaccine, chickens, ducks, H5N1, highly pathogenic

Abbreviations: Ag = antigen; AI = avian influenza; BHI = brain heart infusion; DPC = days postchallenge; EID_{50} = 50% embryo infectious dose; GMT = geometric mean titer; HA = hemagglutinin; HI = hemagglutination inhibition; HP = highly pathogenic; NA = neuraminidase; RRT-PCR = real-time RT-PCR (real-time reverse transcriptase–polymerase chain reaction); SPF = specific-pathogen free

The first case of Asian lineage highly pathogenic (HP) H5N1 avian influenza (AI) virus was isolated in 1996 from a goose in the Guangdong province of China (56,62). A similar virus proceeded to cause an outbreak among poultry in Hong Kong in 1997 (57). Massive culling of all poultry led to the eradication of these viruses, but the Goose/Guangdong-like viruses continued to circulate among ducks in China (44,56). Cauthen *et al.* also demonstrated that H5N1 viruses obtained from cages where geese were housed, in 1999, were nearly identical to the Goose/Guangdong/1/96 virus (5). Evidence of continued circulation of virus in the region included H5N1 from exported Chinese duck meat in 2001, and H5N1 viruses being isolated in live bird markets in Vietnam in 2001 and Hong Kong in 2002 (14,23,50). However, starting late in 2003, an unprecedented spread of the virus occurred in Southeast Asia that eventually moved to Europe, Africa, and the Indian subcontinent (56). These H5N1 viruses have become endemic in several countries in Asia and Africa and variant strains continue to emerge (6,20,35,54,60). Vaccination has been implemented and is still encouraged as part of a control program in poultry in parts of Asia including Vietnam, Indonesia, China, and Egypt (Thanhnien news; 7/15/05) (8).

In addition to preventing clinical disease, a major goal of vaccination against AI (particularly H5 and H7 subtypes), when

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used as part of a control program, is to reduce levels of virus shed into the environment (43,51). Influenza viruses tend to accumulate point mutations in their hemagglutinin (HA) and neuraminidase (NA) surface proteins over time, resulting in antigenic drift, which enhances the ability of the virus to evade the host immune response induced by vaccination or natural infection, allowing higher levels of replication (11,40,61). With the increased amount of virus replication, higher levels of virus shedding by infected birds increase the likelihood of transmission to other susceptible birds or flocks**.** Earlier studies have shown that the closer the HA amino acid sequence of the vaccine virus is to that of the challenge virus, the lower the levels of virus that are shed from the oropharynx (47). Therefore, it is important when selecting a vaccine virus to take into consideration the amino acid sequence similarity between the circulating viruses and the vaccine virus candidates.

For human influenza viruses, the importance of antigenic drift in vaccine seed strains has resulted in the development of the World Health Organization Global Influenza Surveillance Network, whose principal function is to recommend what vaccine seed strains should be included in commercial vaccines. The factors that they evaluate are genetic differences in circulating strains, the prevalence of important variants, and the antigenic differences these viruses have from the current vaccine strains. A fourfold difference in hemagglutination inhibition (HI) titers between antibody against the current vaccine viruses and antigen of reference strains is an indication that the vaccine seed strain needs to be changed to maintain optimal protection from the vaccine (http://www.who.int/csr/disease/influenza/surveillance/en/index.html). Avian influenza viruses at one time were thought to be less susceptible to antigenic drift as it related to vaccine efficacy, at least as measured by morbidity or mortality in vaccinated birds (55). Because cross-protection has been provided by vaccines produced from heterologous viruses (46,52), frequent changing of AI virus vaccine strains was not considered to be necessary (45,47). This difference in human and AI viruses was believed to be a combination of less selection pressure in the birds due to the infrequent vaccination, short production lives of the birds, and the use of strong adjuvants that were commonly used with poultry vaccines (51). However, long-term vaccination for AI in poultry has recently become more common, and in countries like Mexico, where long-term vaccination without eradication of the low-pathogenic H5N2 avian influenza circulating there, a similar degree of antigenic drift as in human influenza viruses seems to occur at the antigenic level in AI viruses (16). In the study by Lee *et al.* in 2004 (18) comparing different Mexican lineage viruses isolated before and during the vaccination campaign, up to a 16-fold difference was seen in HI activity between the vaccine seed strain and the more recent AI isolates. Additionally, amounts of virus shed by vaccinated birds that were challenged with distantly related viruses were comparable to birds that had not been vaccinated (17). The findings from this study underscore the need for frequent evaluation of AI vaccines in their abilities to control viral shedding. Furthermore, it emphasizes the importance of carefully matching vaccine strains with circulating viruses.

In Vietnam, customary duck management practices often allow exposure of domestic ducks to the wild waterfowl population, which enables ample opportunity for AI viruses to infect domestic ducks and potentially to proceed to infect chickens and other poultry (4,10). Because ducks can serve as silent carriers of AI and because they make up a large part of the poultry population in Vietnam, vaccination of ducks has been included in the vaccine regimen (36). Some concern that this increased vaccination pressure may result in faster antigenic drift away from the vaccine strains has been raised. It has been suggested to evaluate vaccine efficacy at least biennially (43) to ensure that optimal levels of protection against clinical disease and viral shedding are met. Although various vaccines' protective abilities against AI challenge in chickens is relatively well understood, less is known about how well AI vaccines perform in ducks. Here, we evaluated the abilities of three commercial vaccines and two experimental vaccines homologous to the challenge strain to protect both chickens and ducks against lethal exposure of highly pathogenic H5N1 viruses that were obtained from chickens and ducks in Vietnam. The challenge viruses selected were classified as clade 2.3.2 or 2.3.4 viruses and were unusually virulent for ducks, as well as being characteristically virulent for chickens (24,30,60).

Table 1. Sequence similarity[A] between commercial vaccines and challenge virus HA1 proteins.

	DK/VN/203/05	CK/VN/209/05	DK/VN/218/05
N28 (H5N2)	87.1	87.7	87.7
Re-1 (H5N1)	92.5	92.5	92.5
Mexican vaccine (H5N2)	83.4	84.9	84.9

[A]The MegAlign program (DNASTAR, Madison, WI) was used to compare amino acid sequences, using the Clustal V alignment algorithm. Percent values shown.

MATERIALS AND METHODS

Viruses. The H5N1 viruses A/Duck/Vietnam/203/05 (DK/VN/203), A/Chicken/Vietnam/209/05 (CK/VN/209), and A/Duck/Vietnam/218/05 (DK/VN/218) were isolated from either ducks or chickens in Vietnam and were obtained from the National Center for Veterinary Diagnosis, Hanoi, Vietnam. The DK/VN/203 virus is a clade 2.3.2 virus and the two latter viruses belong to clade 2.3.4 (24,30,60), and were isolated in northern Vietnam in December of 2005 (30). Isolates were inoculated into the allantoic cavity of embryonating chicken eggs and grown for 24–30 hr at 37 C. Allantoic fluid was harvested, titered as previously described (30), and frozen at −70 C until further use.

Vaccines. Three commercially available vaccines used in Vietnam to control AI were used in this study. These vaccines were generated from the following whole, killed viruses, and contained an oil adjuvant: 1) A/TK/England/N-28/73, subtype H5N2 (referred to as N28); 2) a genetically modified reassortant H5N1 low pathogenic virus, A/Harbin/Re-1/2003 (referred to as Re-1) (31); and 3) A/CK/Mexico/232/94 (H5N2, referred to as Mexican vaccine; Table 1). The first two vaccines were obtained from Vietnam and both have been used in chickens and ducks in all 64 provinces of the country. They were produced by Weike Biological Company, of the Harbin Veterinary Research Institute (Chinese Academy of Agricultural Sciences, Harbin, People's Republic of China). The third vaccine used was from Intervet International/Investigacion Aplicada S.S., Tehuacan, Puebla, Mexico, and its use has been permitted in chickens in Vietnam. The Re-1 vaccine was produced through reverse genetics and derived its HA and NA genes from A/Goose/Guangdong/96 (31). This virus was attenuated by removing the multiple basic amino acids at the HA cleavage site (49). The six internal genes of this recombinant virus were derived from the high-growth A/Puerto Rico/8/34 (PR8) virus.

Two experimental vaccines containing either DK/VN/203 or CK/VN/209 antigen (Ag) were prepared on site, as previously described (41). Briefly, viruses were grown in 10-day-old embryonating chicken eggs for 1 day. Allantoic fluid from eggs infected with one of each particular virus was harvested and pooled. Following inactivation of each virus with 0.1% β-propiolactone (Sigma, St. Louis, MO), HA titers were determined by the HA test to be the following: DK/VN/203: 256 HA units and CK/VN/209: 256 HA units. One part aqueous Ag

(10 ml) was emulsified in four parts (40 ml) oil phase. The oil phase consisted of 36 ml Drakeol 6 VR pharmaceutical grade mineral oil (Penreco, Butler, PA), 3 ml 7.5% sorbitan mono-oleate (Arlacel 80, ICI United States, Inc., Wilmington, DE), and 1 ml 2.5% polysorbate (Tween 80, ICI United States, Inc.). Vaccines produced on site were prepared 5 or 6 days prior to administration, homogenized with the use of a Waring blender (Fisher Scientific International Inc., Hampton, NH) (42), and stored at 4 C.

Evaluation of sequence similarity. Amino acid sequence similarities between vaccine and challenge virus HA1 proteins were compared with the use of the MegAlign program (DNASTAR, Madison, WI). The Clustal V alignment algorithm was used.

Animal experiments. Two-week-old specific-pathogen-free (SPF) white leghorn chickens from our flock at Southeast Poultry Research Laboratory, either 8 or 10 per group, were vaccinated once, subcutaneously in the nape of the neck with one of the three commercial vaccines, as per the company's instructions (0.3 ml of either Chinese vaccine or 0.5 ml of Mexican vaccine), or with 0.5 ml of experimental vaccine (41). Normal allantoic fluid in the form of an oil emulsion vaccine was used as negative control. Two weeks postvaccination, all birds were bled via the wing vein. Three weeks postvaccination, all birds were challenged with 10^6 EID_{50} (50% embryo infectious dose) of either DK/VN/203 or CK/VN/209 viruses in a total volume of 0.2 ml brain heart infusion (BHI) broth per bird, via the choanal slit. Birds were evaluated for clinical signs for 10 days following challenge. Oropharyngeal swabs were taken at 2 and 4 days postchallenge (DPC) for determining viral shedding. Moribund birds that were in pain, or had stopped eating or drinking, were euthanized with 0.2 ml sodium pentobarbital (5 g/ml) per bird. At 10 DPC, all survivors were bled via the wing vein, and then euthanized as described.

One-week-old white Pekin ducks (*Anas platyrhynchos*) obtained from a commercial farm were divided into six groups of 10 birds. Blood samples for serology were collected from the saphenous vein of a representative number of ducks to ensure that the birds were serologically negative for AI, as determined by the agar gel precipitin test (2,26). Ducks were vaccinated once, subcutaneously in the nape of the neck, with one of the three commercial vaccines, as per the company's instructions (0.3 ml of either Chinese vaccine or 0.5 ml of Mexican vaccine). Two groups served as nonvaccinated controls, receiving allantoic fluid in the form of an oil emulsion vaccine. Two weeks postvaccination, blood samples were collected from all ducks for serology. At this same time, the ducks were challenged via the choanal slit with $10^{5.0}$ EID_{50} of DK/VN/203 or DK/VN/218 influenza virus in 0.1 ml. Ducks were observed daily for clinical signs of disease. Oropharyngeal and cloacal swabs were collected at 2, 3, 5, 7, and 11 DPC to determine viral shedding. One duck per group was euthanized with 0.2 ml sodium pentobarbital (5 g/ml) per bird at 3 DPC, and tissues collected for virus detection by quantitative real-time reverse transcriptase–polymerase chain reaction (RRT-PCR) (37). Moribund ducks that were in pain, or that had stopped eating or drinking, were also euthanized. Blood samples were collected at 11 DPC from all surviving ducks. Ducks remaining at the end of the experiment were euthanized as previously described.

Hemagglutination inhibition (HI) test. Hemagglutination inhibition antibody titers against AI were determined by using the HI test (48). Either homologous or heterologous β-propiolactone-inactivated Ag was diluted in phosphate-buffered saline to make a concentration of four HA units. Homologous Ag refers to the same strain of virus used to produce the vaccine. Heterologous Ag refers to any of the three viruses used to produce the vaccines tested in this study and which were not identical to the vaccine virus administered. Fifty microliters of Ag were added per well of a 96-well plate, where test serum was twofold, serially diluted. Plates were incubated for 15 min at room temperature before 0.5% chicken red blood cells were added to each well. Plates were shaken for 15 sec and incubated for 45 min at room temperature. Results were interpreted as the reciprocal of the last well that had complete inhibition of hemagglutination activity.

Determination of viral shedding. Oropharyngeal swab samples from chickens and ducks, and cloacal swab samples from ducks were suspended in 2 ml sterile BHI broth (Sigma-Aldrich, St. Louis, MO) containing 1× antibiotic/antimycotic (Mediatech, Herndon, VA), and frozen at −70 C until RNA extraction. Total viral RNA was extracted using Trizol or MagMAX-96 AI/ND Viral RNA Isolation Kit (Ambion, Inc., Austin, TX), according to the manufacturer's protocol (38). The procedure for RNA isolation was carried out using the KingFisher magnetic particle processing system (Thermo Scientific, Waltham, MA).

RRT-PCR was performed using primers and probe specific for type A avian influenza matrix gene as previously described (37), but with modifications. Two and three nucleotide changes were detected between the DK/VN/203/05 and CK/VN/209/05 matrix genes and the reverse primer created by Spackman *et al.* (37), so new primers were designed specific for these changes. The primer sequences are as follows: DK/VN/203/05 MA-124: 5′-TGCAAAGACATCTTCAAGTTTCTG-3′ and CK/VN/209/05 MA -124: 5′-TGCAAAGACATCCTCAAGTTTCTG-3′. Qiagen (Valencia, CA) OneStep RT-PCR Kit was used under the following conditions: 1× buffer, 3.75 mM $MgCl_2$, 10 pmol each primer, 320 μM each dNTP, 0.12-μM probe, and 13 units Rnase Inhibitor (Promega, Madison, WI). Eight microliters of the RNA sample (mentioned above) and nuclease-free water were added to make a final volume of 25 μl. The reverse transcription reaction consisted of one cycle of 30 min at 50 C, followed by 15 min at 95 C. Forty-five cycles of 1-sec denaturation at 94 C, followed by annealing for 20 sec at 60 C were carried out in the PCR reaction. Both reactions were carried out in a Smart Cycler II (Cepheid, Sunnyvale, CA) real-time PCR machine. Standard curves were generated by taking known amounts of the viruses (as measured in EID_{50}) obtained from egg allantoic fluid, diluting the allantoic fluid serially 10-fold, and then extracting RNA from these virus dilutions. The EID_{50}s of virus from the swab samples were extrapolated from the cycle thresholds by using standard curves generated from the known amounts of RNA of the challenge viruses used (18) and the results presented as EID_{50} equivalents.

Statistical analysis. Hemagglutination inhibition and swab sample data were analyzed with the use of Prism v5 Software package (GraphPad Software Inc., San Diego, CA). One-way ANOVA with Tukey's *post hoc* test was used to analyze $\log_2$ HI titers and viral shedding data. Results with P values <0.05 were considered to be statistically significant. Detection limits of individual RRT-PCR reactions were calculated from the standard curve, setting the cycle threshold value equal to the number of cycles run (38). Samples that were RRT-PCR negative in this study were assigned titer values equal to the detection limit of the RRT-PCR run minus $10^{0.1}$ EID_{50}/ml, as previously described (38).

RESULTS

Vaccine efficacy experiments. Three commercial vaccines that have been used in Vietnam and two experimental vaccines containing viruses homologous to the challenge strains were used. Two of the commercial vaccines (N28 and Re-1) are of the Eurasian lineage of influenza and have sequence similarities to the challenge viruses ranging from 87.1% to 92.5% (Table 1). The third virus is of the North American lineage and is approximately 84% similar to the challenge strains (Table 1).

DK/VN/203/05 challenge in chickens. Two-week-old white leghorn chickens were vaccinated with one of the three commercial vaccines or the homologous vaccine and challenged 3 wk later with DK/VN/203, a clade 2.3.2 virus. Results are shown in Table 2A. All negative control birds died by 2 DPC. Five of eight birds vaccinated with N28 vaccine displayed signs of mild sinusitis, conjunctivitis, and were less active by 2 DPC, but all birds recovered. No clinical symptoms were noted in any vaccinates in the Re-1, Mexican, or homologous vaccine groups.

Total viral RNA was isolated from oropharyngeal swab samples and quantitative RRT-PCR was performed to compare levels of virus shed by birds from the different vaccine groups both 2 and 4 days

Table 2A. Morbidity, mortality, and virus isolation data from chickens vaccinated with inactivated AI vaccine at 2 wk of age and intranasally challenged at 5 wk of age with 10^6 EID_{50} of DK/VN/203/05 H5N1 HPAI virus.

Vaccine group	Morbidity: number ill/total	Mortality: number dead/total (MDT)[D]	Viral RNA detection from O/P[A] swab samples number positive/total (log EID_{50}/ml[BC])	
			2 days postchallenge	4 days postchallenge
Negative control	8/8	8/8 (2)	8/8 (6.2)[a]	N/A
N28	5/8	0/8	5/8 (2.3)[b]	6/8 (2.8)[a]
Re-1	0/8	0/8	3/8 (1.8)[b]	6/8 (2.0)[a]
Mexican	0/8	0/8	6/8 (2.7)[b]	5/8 (2.6)[a]
DK/VN/203/05	0/8	0/8	4/8 (1.7)[b]	3/8 (1.1)[a]

[A]Swab samples were taken from all birds remaining at each time point postchallenge. O/P = oropharyngeal; NA = not applicable.
[B]log EID_{50} equivalents were determined with the use of RRT-PCR specific for type A avian influenza matrix gene (37). Numbers in parentheses are averages of viral titers shed from birds in each group.
[C]Different lowercase superscripts denote significance between treatment groups (within columns; $P < 0.05$) as determined by one-way ANOVA.
[D]MDT = mean death time denoted in days.

following challenge. At 2 DPC, virus shedding from negative control birds was significantly higher than that from all vaccinated birds. At 4 DPC, no significantly different levels of virus were shed between any of the vaccine groups.

We evaluated HI titers with the use of both homologous and heterologous Ag. At 2 wk postvaccination, all vaccinated birds had HI titers (Table 3A). The Mexican vaccine induced the highest HI titers, which averaged to be 70 at 2 wk postvaccination, when homologous Ag was used (Table 3A). Correspondingly, 100% of the birds in these groups did not show any clinical signs (Table 2A). The Re-1 vaccine group had the second-highest titers, with a geometric mean titer (GMT) of 58, 2 wk postvaccination with homologous Ag (Table 3A). The DK/VN/203 and N28 vaccine groups' HI titers were close to 30 at 2 wk postvaccination when homologous Ag was used (Table 3A). An HI titer of 40 is considered to provide consistent and reproducible protection after challenge with any virulent virus of the same subtype (29), but lower titers may also be protective. This was demonstrated in DK/VN/203 and the N28 vaccine groups, where most birds had no or only mild clinical disease (Table 2A). At 10 DPC, HI titers in all surviving birds were at least 40, whether the Ag was homologous or not (Table 3A). As seen with the 2-wk postvaccination sera, HI titers were highest with homologous Ag. Even though the group vaccinated with the Ag that was homologous to the challenge strain did not have the highest HI titers, all of these birds were protected from disease and death (Table 2A).

CK/VN/209/05 challenge in chickens. Two-week-old white leghorn chickens were vaccinated with one of the three commercial vaccines or with the homologous vaccine and challenged 3 wk later with CK/VN/209, a clade 2.3.4 virus. Results are shown in Table 2B. Similar to the DK/VN/203 challenge group, all control birds died by 2 DPC. Five out of 10 birds in the N28 vaccine group displayed signs of depression and conjunctivitis. At 5 DPC, one bird had died and one, which had displayed severe respiratory signs, was euthanized. In the Re-1 vaccine group, two birds died at 7 DPC. Two birds in the homologous vaccine group also died 2 and 3 DPC. None of the deaths from the latter three groups was statistically significant and all but one of the birds had HI titers below the protective level of 40, 2 wk following vaccination. All birds in the Mexican vaccine group were active and eating normally.

Two days following challenge, all vaccinated birds shed significantly less virus than negative control birds (Table 2B). At 4 DPC, birds that were vaccinated with the N28 or Re-1 vaccine shed significantly higher levels of virus than birds that had received the homologous vaccine (Table 2B). There was no correlation between oropharyngeal virus shedding and survival of the birds; not all birds that died were shedding detectable levels of virus, based on RRT-PCR. However, several birds that did shed detectable levels of virus, based on RRT-PCR, survived.

All vaccinated birds seroconverted to AIV at 2 wk following vaccination (Table 3B). The Mexican vaccine induced the highest HI titers (GMT: 108) and the Re-1 vaccine induced the second-highest titers (GMT: 45) 2 wk postvaccination, when homologous Ag was used (Table 3B). Two weeks following vaccination, the group vaccinated with the homologous vaccine had HI titers near 30. However, only one of these birds displayed clinical symptoms

Table 2B. Morbidity, mortality, and virus isolation data from chickens vaccinated with inactivated AI vaccine at 2 wk of age and intranasally challenged at 5 wk of age with 10^6 EID_{50} of CK/VN/209/05 H5N1 HPAI virus.

Vaccine group	Morbidity: number ill/total	Mortality: number dead/total (MDT[D])	Viral RNA detection from O/P[A] swab samples number positive/total (log EID_{50}/ml[BC])	
			2 days postchallenge	4 days postchallenge
Negative control	8/8	8/8 (2)	8/8 (7.2)[a]	N/A
N28	5/10	2/10 (5)	10/10 (4.4)[b]	9/10 (5.2)[a]
Re-1	2/10	2/10 (7)	9/10 (4.1)[b]	8/10 (4.2)[a]
Mexican	0/8	0/8	5/8 (3.0)[b]	6/8 (3.8)[ab]
CK/VN/209/05	2/8	2/8 (2.5)	4/8 (3.0)[b]	2/6 (1.7)[b]

[A]Swab samples were taken from all birds remaining at each time point postchallenge. O/P = oropharyngeal; NA = not applicable.
[B]log EID_{50} equivalents were determined with the use of RRT-PCR specific for type A avian influenza matrix gene (37). Numbers in parentheses are averages of viral titers shed from birds in each group.
[C]Different lowercase superscripts denote significance between treatment groups (within columns; $P < 0.05$) as determined by one-way ANOVA.
[D]MDT = mean death time denoted in days.

Table 3A. HI titers of chickens vaccinated at 2 wk of age and challenged intranasally at 5 wk of age with 10^6 EID_{50} of DK/VN/203/05 H5N1 HPAI virus.[A]

	Range of prechallenge HI titers[B]		Range of postchallenge HI titers[C]	
Vaccine group	Homologous Ag[D]	DK/VN/203/05 Ag	Homologous Ag[D]	DK/VN/203/05 Ag
Negative control	0[a]	0[a]	N/A	N/A
N28	16–64 (30)[b]	4–16 (10)[bc]	32–1,024 (304)[a]	32–512 (152)[a]
Re-1	32–128 (58)[bc]	4–32 (14)[bc]	128–2,048 (832)[a]	32–1,024 (165)[a]
Mexican	32–128 (70)[c]	0–16[E] (7)[b]	256–2,048 (1,323)[a]	64–1,024 (278)[ab]
DK/VN/203/05	8–64 (27)[b]	8–64 (27)[c]	512–2,048 (776)[a]	512–2,048 (776)[b]

[A]Different lowercase superscript letters denote significance between groups (within columns; $P < 0.05$), as determined by one-way ANOVA.
[B]Serum was collected 2 wk postvaccination. In parentheses: geometric mean of HI titers.
[C]Serum was collected from all surviving birds 10 days following challenge. In parentheses: geometric mean of HI titers.
[D]Homologous virus refers to the same strain of virus used to generate the vaccine. Because Goose/Guangdong/1/96 was not available, Goose/Hong Kong/99 was used as homologous Ag for Re-1 group antisera.
[E]One of eight birds did not have detectable levels of HI antibodies against this antigen.

(Table 2B). Birds that had been vaccinated with N28 had significantly lower HI titers than birds vaccinated with the Mexican vaccine, when homologous antigen was used (Table 3B). Similar to the DK/VN/203 challenge study, all surviving birds had HI titers greater than 40 following challenge (Table 3B). At 10 DPC, birds in the Re-1 vaccine group had significantly higher HI titers than those in the homologous vaccine group (Table 3B). As mentioned, all but one of the six vaccinated birds that died following challenge had HI titers less than 40, when homologous Ag was used.

DK/VN/203/05 challenge in ducks. One-week-old Pekin ducks were vaccinated once with one of the three commercial vaccines and challenged 2 wk later with DK/VN/203. Results are shown in Table 4A. All control birds died by 4 DPC. One duck vaccinated with Re-1 presented mild neurological signs including head tilting and incoordination, but continued to eat and drink and appeared alert, and survived until the end of the experiment.

Virus shedding was detected from both the oropharyngeal and cloacal routes from all challenged groups at 2 and 3 DPC. The N28 and Re-1 vaccines induced similar responses in ducks on levels and duration of shedding after challenge. All but one of the ducks vaccinated with the Mexican vaccine stopped shedding by 5 DPC, at least 1 day earlier than with the other two vaccines. Virus was detected from all tissues collected from the ducks euthanized at 3 DPC, demonstrating systemic spread (data not shown).

All vaccinated ducks had seroconverted prior to challenge (Table 5A), when tested against homologous Ag. Birds in the Re-1 vaccine group had significantly higher prechallenge titers than those in the N28 or Mexican vaccine groups (Table 5A). All groups' HI antibody titers were undetectable, prior to challenge, when heterologous antigen was used (Table 5A). No significant differences were detected between vaccine groups when homologous antigen was used on serum collected 11 DPC (Table 5A). However, ducks vaccinated with the Mexican vaccine had significantly higher HI titers than those vaccinated with N28 when heterologous Ag was used (Table 5A).

DK/VN/218/05 challenge in ducks. One-week-old Pekin ducks were vaccinated once with one of the three commercial vaccines and challenged 2 wk later with DK/VN/218. Results are shown in Table 4B. Negative control birds died in less than 3 DPC. All but one of the immunized ducks were protected against mortality upon challenge. This duck was vaccinated with N28 and it died 6 days after infection. This duck presented neurological signs as described above.

Compared to ducks challenged with the DK/VN/203 vaccine, those challenged with DK/VN/218 generally shed virus for a longer time period. Viral shedding was detected from both oropharyngeal and cloacal swabs through at least 5 days, and many continued shedding virus at 11 DPC. In the DK/VN/218 challenge group, the Mexican vaccine curtailed oropharyngeal shedding by at least 5 days, compared to the other two vaccines. Similar to the DK/VN/203/05 challenge birds, virus was detected in all tissues collected from the ducks euthanized at 3 DPC (data not shown).

Prior to challenge, all vaccinated birds had HI titers (Table 5B). No significant differences in HI titers were seen between any of the vaccinated groups prior to challenge when homologous Ag was used. No HI antibodies were detectable when heterologous Ag to the vaccine virus was used (Table 5B). At 11 DPC, when homologous Ag was used, no significant differences were seen between vaccine

Table 3B. HI titers of chickens vaccinated at 2 wk of age and challenged intranasally at 5 wk of age with 10^6 EID_{50} of CK/VN/209/05 H5N1 HPAI virus.[A]

	Range of prechallenge HI titers[B]		Range of postchallenge HI titers[C]	
Vaccine group	Homologous Ag[D]	CK/VN/209/05 Ag	Homologous Ag[D]	CK/VN/209/05 Ag
Negative control	0[a]	0[a]	N/A	N/A
N28	8–128 (24)[b]	0–32[E] (13)[b]	128–2,048 (861)[ab]	128–256 (194)[a]
Re-1	2–256 (45)[bc]	4–64 (18)[b]	512–4,096 (1,722)[a]	64–512 (181)[a]
Mexican	32–512 (108)[c]	4–32 (13)[b]	512–4,096 (891)[ab]	64–1,024 (194)[a]
CK/VN/209/05	4–128 (35)[bc]	4–128 (35)[b]	256–512 (362)[b]	256–512 (362)[a]

[A]Different lowercase superscript letters denote significance between groups (within columns; $P < 0.05$), as determined by one-way ANOVA.
[B]Serum was collected 2 wk postvaccination. In parentheses: geometric mean of HI titers.
[C]Serum was collected from all surviving birds 10 days following challenge. In parentheses: geometric mean of HI titers.
[D]Homologous virus refers to the same strain of virus used to generate the vaccine. Because Goose/Guangdong/1/96 was not available, Goose/Hong Kong/99 was used as homologous Ag for Re-1 group antisera.
[E]One of 10 birds did not have detectable levels of HI antibodies against this antigen.

Table 4A. Mortality and virus detection data from Pekin ducks vaccinated with inactivated AI vaccines at 1 wk of age and intranasally challenged at 3 wk of age with 10^5 EID_{50} of DK/VN/203 H5N1 HPAI virus.

		Viral RNA detection from swab samples[A] (log EID_{50}/ml)									
	Mortality: number dead/ total (MDT)[B]	2 DPC		3 DPC		5 DPC		7 DPC		11 DPC	
Vaccine group		O/P[C]	Cloacal	O/P	Cloacal	O/P	Cloacal	O/P	Cloacal	O/P	Cloacal
Negative control	10/10 (3.7)[D]	9/10 (4.4)	5/10 (2.8)	6/6 (4.6)	6/6 (3.2)	—	—	—	—	—	—
N28	0/10	4/10 (3.5)	2/10 (2.9)	5/10 (3.7)	1/10 (3.1)	9/9 (2.9)	4/9 (2.8)	0/9	0/9	0/9	0/9
Re-1	0/10	4/10 (3.1)	1/10 (2.5)	9/10 (3.2)	9/10 (3.1)	5/9 (2.8)	2/9 (3.1)	0/9	0/9	0/9	0/9
Mexican	0/10	3/10 (3.2)	2/10 (4.5)	10/10 (3.3)	9/10 (3.2)	1/9 (3.3)	0/9	0/9	0/9	0/9	0/9

[A]log EID_{50} equivalents were determined with the use of RRT-PCR specific for type A avian influenza matrix gene (37).
[B]MDT = mean death time denoted in days.
[C]O/P = oropharyngeal.
[D]Number of birds shedding/total number of birds in group. In parentheses: average viral titers from birds in each group.

groups. Ducks vaccinated with the Mexican vaccine had significantly higher HI titers than those vaccinated with Re-1 and then challenged with DK/VN/218/05, when challenge strain viral Ag was used (Table 5B).

DISCUSSION

When used as part of an effective control strategy against AI, vaccination should not only prevent clinical signs and illness, but also significantly reduce the amount of viral shedding that could be a source of infection for other birds (19,43). The Asian lineage of H5N1 AI first caused disease outbreaks in poultry in Vietnam at the start of 2004 (58), and it spread widely across the country. In 2005, a campaign to vaccinate 220 million domestic fowl with the commercial vaccines tested in this study was launched in an attempt to control the outbreak (http://www.globalsecurity.org/security/library/news/2009/02/sec-090211-irin01.htm) (25). Between 2005 and 2006, the two Chinese vaccines (N28 and Re-1) were used in both chickens and ducks, while the Mexican vaccine was used exclusively in chickens (personal communication). It appeared to be a success over the next year by greatly reducing the number of reported outbreaks and human infections in Vietnam (59). During the latter part of 2006, H5N1 viruses were reported in nonvaccinated, asymptomatic ducks, upon routine surveillance (59). Even though vaccination is still being used in Vietnam, poultry outbreaks as well as human cases continue to be reported (59). The factor of low immunity rate in poultry, because of the difficulty to vaccinate and booster poultry populations, is thought to contribute to the resurgence of the disease. In addition, the viruses in Vietnam continue to change both by antigenic drift as well as new variants being introduced from other countries in the region. One of the goals of this study was to evaluate if the change of the lineage of virus circulating in northern Vietnam, from clade 1 to clade 2.3, contributed to the increase of poultry outbreaks in spite of the continued efforts at vaccination. For this, we evaluated the level of protection obtained with three widely available commercial vaccines in chickens and ducks after challenge with viruses representative of clade 2.3 strains circulating in Vietnam.

Although H5N1 HPAI viruses display virulent phenotypes in chickens, ducks may become infected and shed virus without presenting any signs of illness (1,7,15,26,27,28,33). Therefore, ducks have been linked to transmitting AI to poultry (34) by ''silently'' spreading virus, contributing to its circulation and further propagation among poultry. As a result, the H5N1 HPAI viruses continue to threaten both human and veterinary/poultry health. On the other hand, some HPAI viruses circulating in Vietnam have shown to produce high mortality in domestic ducks (30), directly affecting this important segment of this country's poultry industry. The duck-raising practices in Vietnam include the production of free-range ducks, which, because of the low biosecurity inherent with this production practice, poses a high risk of spread and maintenance of H5N1 in the country (21). Consequently, outbreaks continue to occur in nonvaccinated ducks (9,59). If efficacious vaccines could be given to ducks in this production system, it could significantly improve the control of AI.

Because of AI's tendency to drift antigenically, AI vaccines should be tested periodically to ensure sufficient protection from clinical disease and virus shedding (32,43). The challenge viruses for this study were not only highly pathogenic in chickens, but unusually

Table 4B. Mortality and virus detection data from Pekin ducks vaccinated with inactivated AI vaccines at 1 wk of age and intranasally challenged at 3 wk of age with 10^5 EID_{50} of DK/VN/218 H5N1 HPAI virus.

		Viral RNA detection from swab samples[A]									
	Mortality: number dead/ total (MDT)[B]	2 DPC		3 DPC		5 DPC		7 DPC		11 DPC	
Vaccine group		O/P[C]	Cloacal	O/P	Cloacal	O/P	Cloacal	O/P	Cloacal	O/P	Cloacal
Negative control	10/10 (2.1)[D]	10/10 (3.4)	9/10 (4.9)	1/1 (4.8)	1/1 (2.9)	—	—	—	—	—	—
N28	1/10 (6)	5/10 (3.2)	1/10 (3.6)	4/10 (3.1)	2/10 (3.4)	4/9 (2.8)	7/9 (2.8)	4/8 (2.9)	8/8 (2.9)	5/8 (2.7)	8/8 (2.7)
Re-1	0/10	4/10 (2.8)	1/10 (2.6)	3/10 (3.7)	2/10 (2.5)	8/9 (2.9)	5/9 (2.6)	5/9 (2.8)	0/9	6/9 (2.8)	0/9
Mexican	0/10	3/10 (2.9)	1/10 (5.0)	4/10 (3.0)	5/10 (3.2)	4/9 (2.7)	7/9 (2.7)	0/9	0/9	0/9	0/9

[A]log EID_{50} equivalents were determined with the use of RRT-PCR specific for type A avian influenza matrix gene (37).
[B]MDT = mean death time denoted in days.
[C]O/P = oropharyngeal.
[D]Number of birds shedding/total number of birds in group. In parentheses: average viral titers from birds in each group.

Table 5A. Hemagglutination inhibition (HI) titers of ducks vaccinated at 1 wk of age and challenged intranasally at 3 wk of age with 10^5 EID_{50} of DK/VN/203/05 H5N1 HPAI virus.[A]

Vaccine group	Range of prechallenge HI titer[B]		Range of postchallenge HI titer[C]	
	Homologous Ag[D]	DK/VN203/05 Ag	Homologous Ag[D]	DK/VN/203/05 Ag
Negative control	0[a]	0	N/A[E]	N/A
N28	16–32 (17)[b]	0	64–256 (166)[a]	0–16[F] (3)[a]
Re–1	32–128 (42)[c]	0	512–1,024 (446)[a]	0–32[G] (7)[ab]
Mexican	16–128 (23)[b]	0	128–1,024 (276)[a]	8–64 (16)[b]

[A]Different lowercase superscript letters denote significance between groups (within columns; $P < 0.05$), as determined by one-way ANOVA.
[B]Serum samples were taken 2 wk postvaccination. In parentheses: geometric mean of HI titers.
[C]Serum samples were collected 11 days postinfection.
[D]Homologous antigen refers to a virus strain identical to the virus used to generate the vaccine.
[E]NA = not applicable.
[F]Four out of nine birds did not have detectable levels of HI antibodies against this antigen.
[G]Two out of eight birds did not have detectable levels of HI antibodies against this antigen.

virulent for ducks, as well (30). Specifically for ducks, an increase in tissue tropism, lesion severity, viral replication, and one of the shortest mean death times reported in both 2- and 5-wk-old Pekin ducks was observed with these viruses, as compared to previous H5N1 viruses tested in ducks (30). The level of protection rendered by the vaccines was influenced by the virus that served as the challenge strain. Though both of the groups of viruses were extremely virulent in chickens and ducks, the clade 2.3.4 viruses (CK/VN/209 and DK/VN/218) appeared more pathogenic than the clade 2.3.2 (DK/VN/203) virus (30), and caused higher morbidity and virus shedding in ducks.

Hemagglutination inhibition titers are commonly used to predict levels of protection against viral infection and disease in vaccinated birds. In chickens, the Mexican vaccine surpassed the others in its ability to induce high levels of HI antibodies (Table 3A,B) and to protect the birds from clinical disease (Table 2A,B). This is quite interesting because, compared to the other vaccine virus HA sequences, it has the lowest sequence similarity with the challenge viruses. Similar results were seen by Swayne *et al.* and Veits *et al.,* when testing the efficacy of H5N2 vaccines to protect chickens against HPAI viruses that were less than ideally matched to the vaccine viruses (46,53). Such remarkable immunogenicity could possibly be attributed to the proprietary adjuvant used in the formulation of the Mexican vaccine, the antigen mass used in the vaccine, or the inherent antigenicity of the hemagglutinin protein itself (45). With regards to shedding, chickens vaccinated with the CK/VN/209/05 vaccine (and challenged with the same virus) shed significantly lower amounts of virus from the respiratory tract, compared to the N28 vaccine, which was a low pathogenic turkey virus of Eurasian lineage isolated in 1973. In addition, several of the birds vaccinated with this older virus also displayed clinical symptoms. Another study tested the Re-1 vaccine in chickens and demonstrated, as did we, that all of the vaccinated birds were completely protected from disease and death, upon challenge with either homologous virus or heterologous viruses from 2004 (49). There was also virus detected in oropharyngeal swab samples from some of the vaccinated birds (49), similar to our findings.

Compilation of the data produced in chickens indicates that the sequence similarity is not the sole determining factor for predicting a vaccine's protective potential against disease or viral shedding. If the antibody titers are high enough to a subtype, protection from morbidity may be achieved, regardless of the differences in genetic relatedness of the vaccine and challenge viruses if the viruses are of the same subtype. It also appeared that even if the HI titers, using homologous Ag, prior to challenge were not quite at the typical protective level of 40, clinical protection was still observed. Additionally, there did not appear to be a clear correlation between HI titer and level of viral shedding.

In a previous duck study, a two-dose vaccination program starting in ducks at 1 day of age, followed by a booster at 4 wk of age, was used because of its compatibility with the duck husbandry practices in Asia, and was shown to be effective (3). It is important to vaccinate ducks at an early age to try to provide immunity as early as possible, but also because after the ducks are released into the fields, it becomes much more difficult to vaccinate them. We chose to use 1-wk-old ducks on a single-dose regimen to see if, when vaccinated at this age, they would obtain good protection and a reduction in virus shedding after challenge. We also chose to use 1-wk-old ducks because with increased vaccination of poultry in Vietnam, day-old birds may have maternal antibodies that could

Table 5B. Hemagglutination inhibition (HI) titers of ducks vaccinated at 1 wk of age and challenged intranasally at 3 wk of age with 10^5 EID_{50} of DK/VN/218/05 H5N1 HPAI virus.[A]

Vaccine group	Range of prechallenge HI titer[B]		Range of postchallenge HI titer[C]	
	Homologous Ag[D]	DK/VN218/05 Ag	Homologous Ag[D]	DK/VN/218/05 Ag
Negative control	0[a]	0	N/A[E]	N/A
N28	16–32 (20)[b]	0	256–1,024 (380)[a]	8–32 (17)[ab]
Re-1	16–128 (34)[b]	0	128–1,024 (474)[a]	0–32[F] (7)[a]
Mexican	16–256 (26)[b]	0	128–1,024 (406)[a]	16–128 (64)[b]

[A]Different lowercase superscript letters denote significance between groups (within columns; $P < 0.05$), as determined by one-way ANOVA.
[B]Serum samples were taken 2 wk postvaccination. Titers are expressed as geometric mean titers.
[C]Serum samples were collected 11 days postinfection.
[D]Homologous antigen refers to a virus strain identical to the virus used to generate the vaccine.
[E]NA = not applicable.
[F]Three out of nine birds did not have detectable levels of HI antibodies against this antigen.

interfere with vaccine efficacy, and 1-wk vaccination is potentially a suitable compromise with ease of vaccination and less interference by maternal antibody.

Other duck studies involving various vaccination regimens followed by challenge have also demonstrated clinical protection and reduced virus shedding (12,22,39,40,52). However, these results were obtained, as ours, in laboratory settings. It is important to keep this in mind when applying experimental vaccine data to the field. Experimental data can not be directly extrapolated to the field setting because of differences in circumstances between the two. Unlike poultry raised in the field, the experimental animals do not have preexisting immunity to AI from maternal antibodies or prior AI infection and their immune systems are not compromised by other unrelated pathogens, which may be concomitantly circulating among flocks in a field setting. In any case, vaccination is not likely to prevent infection and provide sterilizing immunity. In a previous study, prevention of tracheal and cloacal shedding was achieved when a large dose (1 μg) of antigen was administered (13). Unfortunately, the large quantities of antigen or adjuvant required to induce such a potent immune response may be greater than could be realistically administered in the field for reasons of cost. With an appropriate vaccination program, however, shedding of infectious virus into the environment could be reduced to a minimum and consequently prevent transmission.

Kim *et al.* (13) tested vaccines that contained the HAs of either clade 1, clade 2.2, or clade 2.3.4 viruses, in their abilities to protect SPF white Pekin ducks from an extremely virulent H5N1 virus, Duck/Laos/25/06. Despite low or undetectable HI titers, all of the challenged, vaccinated birds were completely protected from morbidity and mortality after one vaccination (13). Regardless of the time point, the HI titers of vaccinated ducks in our study were much lower than those of chickens (Tables 3A,B, 5A,B). The results of their study support our findings that even if the humoral immune response to the vaccine viruses is not always detectable in ducks, the immune response may still be protective.

At 10 and 11 DPC, HI titers in all surviving birds, chickens and ducks alike, were at least 40, when homologous Ag was used (Tables 3A,B, 5A,B). However, compared to titers produced when heterologous Ag was used, the homologous HI titers were between 4- and 16-fold higher. This suggests that following challenge, the antibodies produced were not only against the challenge strain, but were the result of a memory response against the vaccine virus, as well.

Based on our results, the vaccines tested in this study provided both chickens and ducks protection from disease and reduced viral shedding, upon challenge with either of two different isolated clade 2.3 H5N1 highly pathogenic AI viruses from Vietnam. Though most birds vaccinated with the N28 vaccine did have clinical protection from virulent challenge, as compared to the nonvaccinated birds, the HI titers prechallenge were the lowest of the vaccines tested and the reduction of viral shedding was marginal. This vaccine would seem to be a poor option for a vaccine program, although the reasons for a poor response from this antigen were not fully investigated. The adjuvant used was assumed to be similar to the Re-1 vaccine because it was made by the same manufacturer, but differences in antigen mass, antigenicity of the hemagglutinin, or antigenic differences based both on HI data and sequence similarity may have all contributed to the poorer results. This vaccine would not be recommended for further use. The Re-1 and the Mexican lineage vaccines, which have continued to be used since 2007, still appear to provide good protection from challenge, but antigenic variability based on HI data and sequence similarity raises concern that vaccines made with these viruses will lose protectiveness as the field viruses continue to drift. The need to update vaccine seed strains is critical if optimal protection from vaccination is to be realized. However, good surveillance is needed to understand what viruses are circulating in a region or country. Since December 2005, in northern Vietnam, both clade 2.3.2 and 2.3.4 viruses were cocirculating, and therefore a single vaccine is unlikely to provide optimal protection. Availability of vaccines currently is problematic, not only because of antigenic drift, but also because of differential immune responses to vaccines in chickens as well as in individual duck species (Pantin-Jackwood and Suarez, unpubl. data). It will be useful to continue evaluating the current vaccines not only in chickens, but various species of ducks, also.

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